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Rapid detection of SARS-CoV-2 with Cas13

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Summary

To combat disease outbreaks such as the COVID-19 pandemic, flexible diagnostics for rapid viral detection are greatly needed. We report a nucleic acid test that integrates distinct mechanisms of DNA and RNA amplification optimized for high sensitivity and rapid kinetics, linked to Cas13 detection for specificity. We paired this workflow, termed Diagnostics with Coronavirus Enzymatic Reporting (DISCoVER), with extraction-free sample lysis using shelf-stable reagents that are widely available at low cost. DISCoVER has been validated on saliva samples to incentivize frequent testing for more widespread community surveillance and robustly detected attomolar levels of SARS-CoV-2 within 30 minutes, while avoiding false positives in virus-negative saliva. Furthermore, DISCoVER is compatible with multiplexed CRISPR probes to enable simultaneous detection of a human gene control or alternative pathogens.

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Introduction

Rapid nucleic acid detection is a critical component of a robust testing infrastructure for controlling disease transmission. To date, well over 1 million deaths from coronavirus disease 2019 (COVID-19) have been attributed to over 50 million infections of its causal virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Upon infection, individuals undergo an asymptomatic incubation phase when the virus is seeding and replicating in the lungs and nasal cilliated airway cells, followed by exponential viral production and reports of substantial pre-symptomatic and asymptomatic transmission (Harrison et al. 2020; Bai et al. 2020). By symptom onset, viral loads in the upper respiratory tract are already declining from peak levels and accompanied by a steep drop in nucleic acid test positivity (Wölfel et al. 2020; Zhao et al. 2020).

The challenges of a high asymptomatic infection rate (Lavezzo et al. 2020), insufficient testing, and the narrow time window when molecular tests have high sensitivity have been exacerbated by the long sample-to-answer time of the centralized diagnostic laboratory model. Laboratory-developed tests such as quantitative PCR (qPCR) are conducted in facilities that require labor-intensive personnel and equipment infrastructure for sample accessioning, nucleic acid extraction, thermocycling, and data analysis.

Fast, frequent and point-of-care testing, such as upon entry into the workplace or classroom, is postulated to be an effective way to break the chain of transmission (Larremore et al. 2020). There is also tremendous potential for community surveillance testing to augment clinical workflows, where positive cases are confirmed by referral to a more constrained supply of clinical-grade tests. Alternative sampling methods and test technologies can also help diversify the diagnostic supply chain, as the standard pipeline for clinical testing has proven vulnerable to reagent constraints such as RNA extraction kit or swab shortages (Vandenberg et al. 2020).

We therefore sought to develop a method that did not require RNA extraction or upper respiratory tract swabs. qPCR-based assays have been previously developed with direct sample spike-in, and typically use high temperature for sample lysis (Bloom et al. 2020). Other approaches have exploited chaotropic agents, chemical reduction, and RNase inhibitors (Myhrvold et al. 2018). To reduce swab usage, saliva samples can be collected while minimizing

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contact between healthcare workers and patients, improving safety and decreasing PPE demand. Saliva sampling is reported to have 97% concordance with nasopharyngeal (NP) swabs in RT-qPCR detection, indicating it can be a reliable method for detection (Iwasaki et al. 2020; Anne Louise Wyllie et al. 2020)).

Coupled with direct lysis and saliva sampling, CRISPR-based detection and isothermal amplification have significant potential for point-of-care diagnostics. CRISPR enzyme-based nucleic acid detection relies on the guide RNA-dependent activation of Cas13 or Cas12 nucleases to induce non-specific ssRNA or ssDNA nuclease activity, respectively, in order to cleave and release a caged reporter molecule (East-Seletsky et al. 2016; Gootenberg et al. 2017; Chen et al. 2018). The released reporter can be detected with fluorescent or lateral flow assays to read out the test result. CRISPR-based detection is highly specific, but Cas13 nucleases alone can take up to two hours to reach attomolar sensitivity for diagnostic applications (Fozouni et al. 2020). In contrast, loop-mediated isothermal amplification (LAMP) performs highly sensitive nucleic acid amplification in under 20 minutes with attomolar limits of detection (LODs) (Nagamine, Hase, and Notomi 2002). However, despite the sensitivity and speed of LAMP, such isothermal methods are often prone to non-specific amplification (Hardinge and Murray 2019).

To address these challenges, we report DISCoVER (DiagnosticS with Coronavirus Enzymatic Reporting), an RNA extraction-free SARS-CoV-2 test that combines two distinct amplification mechanisms for sensitivity with a Cas13-mediated probe for specificity (**Figure 1**). Following direct lysis and inactivation of saliva samples via denaturation and reduction, we combined LAMP with T7 transcription to provide an additional layer of RNA-based target amplification after LAMP amplification.

DISCoVER incorporates a 20-30 minute amplification step followed by Cas13 readout in under 5 minutes, and achieves attomolar sensitivity on saliva samples. Compared to other CRISPR-based tests such as DETECTR and STOPCovid V2 (Broughton et al. 2020; Joung et al. 2020), which incorporate LAMP with Cas12-based detection, DISCoVER does not employ a sample extraction or purification step. We validate DISCoVER on a saliva-based sample matrix with live SARS-CoV-2 virus, multiplexed with a human gene process control, to enable facile development of point-of-care COVID-19 diagnostics.

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Results

We first sought to compare the nucleic acid detection properties of Cas13 and Cas12 enzymes, assessing the reporter cleavage activity of *Leptotrichia buccalis* Cas13a (LbuCas13a) and *Lachnospiraceae* bacterium ND2006 Cas12a (LbCas12a) with matching guide RNA spacer sequences in a dilution series of their corresponding synthetic activator molecules (**Figure 2A**). Cas13 detection was significantly more rapid than Cas12, with a mean of 80-fold and 20-fold faster time to half-maximum fluorescence (**Figure 2B**) at 1 nM and 100 pM activator concentrations, respectively. However, the limit of detection of LbuCas13a is still in the femtomolar range after 60 minutes (East-Seletsky et al. 2017), which is already beyond the maximum sample-to-answer time that is likely to be relevant for a point-of-care assay.

To achieve attomolar sensitivity within 30-40 minutes, we chose LAMP as a cost-effective and rapid method for isothermal amplification. LAMP employs a reverse transcriptase, a strand displacing DNA polymerase, and three primer pairs to convert viral RNA to DNA substrates for LAMP. We screened nine LAMP primer sets targeting distinct regions across the length of the SARS-CoV-2 genome (**Figure 2C, Supplementary Table 1**). When targeted to SARS-CoV-2 genomic RNA fragments at 100 copies/uL, all sets resulted in positive LAMP signals. Maximum fluorescence was reached within 20 minutes (**Figure 2D**) for all primer sets, and time-to-threshold was determined via the single threshold Cq determination mode as indicated. LAMP primer sets targeting Orf1ab Set 1, N Set 1, and N Set 2 consistently amplified in under 15 minutes (**Figure 2E**).

Each LAMP primer set resulted in a no-template control (NTC) signal, albeit with a delay relative to the positive condition containing viral RNA (**Figure 2D**). This high false-positive rate, potentially due to primer dimer formation, can in principle be reduced with a second probe that selectively recognizes the amplified nucleic acid sequence. We therefore sought to combine the sensitivity of LAMP detection with the specificity of Cas13 target recognition.

To avoid Cas13 detection of non-specific amplification, its guide RNAs must have minimal to no sequence overlap with the primer sequences. Due to the complexity of LAMP concatemerization, LAMP primers are highly overlapping and are designed with short amplicons to increase the

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reaction speed (Notomi 2000). Our LAMP primer sets generated amplicons ranging from 1-60 nt, and so N Set 1, targeting the SARS-CoV-2 N gene, was chosen for further use due to its low time-to-threshold and an amplicon size capable of accommodating Cas13 guide RNAs (**Figure 2E, Supplemental Table 1**). We next performed a dilution series of genomic viral RNA and determined the N Set 1 LOD to be 25 copies/ µL (**Figure 2F**), comparable with previous studies that report LODs between 10-100 copies/ µL (Dao Thi et al. 2020; El-Tholoth, Bau, and Song 2020; Rabe and Cepko 2020).

Because Cas13 targets single-stranded RNA (**Figure 2A**), while LAMP amplifies DNA substrates, we reasoned that transcription of the LAMP products would enable substrate compatibility. T7 RNA polymerase promoter sequences were incorporated into the LAMP primer sequences to enable subsequent transcription and Cas13 detection. We termed this LAMP amplification to RNA (rLAMP). LAMP employs three primer pairs: forward and backward outer primers (F3/B3) for initial target strand displacement, forward and backward inner primers (FIP/BIP) to form the core LAMP stem-loop structure, and forward and reverse loop primers (Floop/Bloop) for an additional layer of loop-based amplification (**Supplementary Figure 1**). Through multiple iterations of primer binding and extension, these stem-loop structures amplify into concatemers composed of inverted repeats of the target sequence (**Figure 3A**).

To enable rLAMP, we systematically tested the insertion of T7 promoter sequences in three different regions of LAMP primers - on the 5’ end of the FIP and BIP primers (5’FIP/5’BIP), in the middle of the FIP and BIP primers (mFIP/mBIP), and on the 5’ end of the loop primers (FLoop/BLoop) (**Figure 3B**). Addition of the T7 promoter did not greatly affect rLAMP time-to-threshold of N Set 1, given viral genomic RNA at 100 copies/ µL (**Figure 3C**). To confirm the target sequences were specifically amplified, we performed restriction enzyme digestion on the LAMP products using AfeI, which digests in the Cas13 guide RNA target region within the rLAMP amplicon (**Supplementary Figure 2A, 2B**). Lack of AfeI digestion in all NTC conditions confirmed that NTC signal is non-specific amplification lacking the guide-matching target sequence.

To test whether the T7 promoter was properly incorporated and functional in the rLAMP amplicon, we next performed *in vitro* transcription with T7 RNA polymerase. Denaturing PAGE

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analysis indicated that the virus template and NTC conditions resulted in significant RNA transcription for all primer sets (**Figure 3D**). AfeI digestion of the mBIP rLAMP product should produce a single 147 nt product containing the T7 promoter (**Supplementary Figure 2A**). Subsequent T7 transcription resulted in the expected 85 nt RNA product (**Figure 3D**).

Next, we optimized buffer conditions to support T7 transcription and Cas13 detection in a single step, followed by systematic screening of Cas13 cleavage activity in the presence of rLAMP products containing T7 promoter insertions in different rLAMP amplicons. We determined that the middle of the BIP primer (mBIP) insertion position resulted in the fastest detection and therefore chose this primer set for further studies (**Supplementary Figure 3**). Cas13 rapidly detects the rLAMP amplicon with viral template, while avoiding detection of non-specific NTC amplicons, achieving over 10-fold change in signal over the NTC background in under two minutes (**Figure 3E**). Saturating signal was observed within five minutes, reaching over 40-fold signal over background.

To confirm replicability of the DISCoVER pipeline, we tested Cas13 detection on 8 replicates of mBIP rLAMP reactions with activator RNA included at a concentration of 100 copies/ µL in a 20 µL reaction. All 8 replicates resulted in over 25 fold-change in signal over NTC, which is stable well beyond the 5 minute detection employed here (**Figure 3F**).

With an amplification and detection protocol in place, we next optimized sample processing to establish a simple protocol of heat paired with chemical reagents to promote viral inactivation and dampen the activity of RNA-degrading nucleases present in saliva (Ostheim et al. 2020). We assayed two concentrations of the shelf-stable reducing agent TCEP (Tris(2-carboxyethyl)phosphine) paired with the ion chelator EDTA (ethylenediaminetetraacetic acid) (Rabe and Cepko 2020; Myhrvold et al. 2018), commercially available reagents such as QuickExtract buffers containing detergents and proteinase, and DNA/RNA shield containing chaotropic guanidine thiocyanate.

To test the compatibility of these inactivating reagents with rLAMP, we created mock positive saliva samples by adding the reagents to heat-treated saliva at 75 °C (Chin et al. 2020) and adding SARS-CoV-2 genomic RNA at two different concentrations: 1000 cp/ µL and 200 cp/uL.

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In the absence of inactivating reagents, we were unable to detect any rLAMP signal, suggesting degradation of RNA in saliva by endogenous RNases present in the sample. In contrast, genomic RNA without saliva was rapidly amplified in under 15 min. Only the low concentration condition of TCEP-EDTA was capable of protecting target RNA and preserving rLAMP sensitivity in all 4 replicates (**Figure 4A**). This reagent cocktail simultaneously breaks protein disulfide bonds and sequesters divalent cations. Its activity is expected to dampen RNase activity while simultaneously disrupting mucin gel formation, reducing variable saliva viscosity for simpler sample processing (Meldrum et al. 2018; Tabachnik, Blackburn, and Cerami 1981).

Finally, we used the guidelines provided by the FDA for Emergency Use Authorization (EUA) in May 2020 to determine the analytical sensitivity and specificity for DISCoVER on saliva samples (Food and Drug Administration, 2020). To determine the limit of detection, viral stocks were serially diluted in media and quantified with RT-qPCR relative to a standard curve generated from synthetic genomic RNA. These known concentrations of virus were spiked into negative saliva samples collected before November 2019 in BSL3 conditions and run through the DISCoVER workflow (**Figure 4B**). We performed 20 DISCoVER replicates for a range of virus concentrations, determining 40 cp/ µL of virus in directly lysed saliva (**Figure 4C**) to be the lowest concentration tested where at least 19/20 replicates amplified successfully. To assay DISCoVER specificity, saliva samples from 30 different individuals negative for SARS-CoV-2 were tested without any false positive signal (**Figure 4D**).

In point-of-care settings, successful sample inactivation and lysis would ideally be confirmed by an internal process control (**Figure 4E**). This is particularly important for successful saliva-based population screening, as some individuals can have high viscosity or mucin gel loads in their samples. To achieve this, we multiplexed detection of the SARS-CoV-2 N gene and the human RNase P gene in the rLAMP amplification step. Cas13 detection of RNase P on saliva samples also reached saturation within 5 minutes, enabling confirmation of saliva RNase inactivation and viral lysis (**Figure 4F**).

Discussion

Here, we have demonstrated an RNA extraction-free workflow for the facile detection of SARS-CoV-2 virus. DISCoVER's combination of a 20-30 minute LAMP step followed by T7

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transcription and Cas13-based detection creates a rapid testing protocol with attomolar sensitivity and high specificity. As each LAMP product can serve as a substrate for transcription initiation, peak Cas13 signal occurs in under five minutes due to rapid generation of nanomolar substrate concentrations (**Supplementary Figure 3**). The combination of sensitive nucleic acid amplification with CRISPR-mediated specificity and programmability enables flexible diagnostics for diverse pathogen detection.

We demonstrate the DISCoVER system on unextracted saliva, with the aim of enabling the first key steps for a point-of care diagnostic. A saliva-based assay may not require medical personnel for sample collection, as is preferable for nasopharyngeal swabs, and the increased comfort of sample collection will likely incentivize patient compliance and commitment to frequent testing. It has also been shown that saliva is a reliable sample matrix for asymptomatic testing in community surveillance, as saliva samples have comparable viral titers to NP swabs (Wyllie et al. 2020). To diversify sampling supply chain, DISCoVER can also be applied to other samples such as nasopharyngeal swabs and self-administered anterior nares swabs.

In comparison with other CRISPR detection methods such as DETECTR and STOPCovid V2, DISCoVER does not employ a sample extraction or purification step (Broughton et al. 2020; Joung et al. 2020), eliminating reliance on commercial RNA extraction kits. The direct lysis method employed here exploits common reagents for chemical reduction and ion chelation that are simple, widely available at low cost, and stable at room temperature. DISCoVER maintains attomolar sensitivity in comparison to other CRISPR based detection assays that include viral RNA extraction and purification (Broughton et al. 2020; Patchsung et al. 2020; Fozouni et al. 2020).

Finally, we demonstrate multiplexed SARS-CoV-2 detection with a human internal control during the DISCoVER amplification stage. This can be adapted for multi-color detection using Cas enzymes with orthogonal cleavage motifs, acting on reporters for distinct fluorescent channels (Gootenberg et al. 2018). Higher-order multiplexing can be further developed for influenza types A and B and other common respiratory viruses that would be desirable to detect in a single test. The inherent ability of the core enzymes in DISCoVER to convert between any RNA and DNA sequence, implies that any pathogen that is inactivated and lysed by our protocol

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can be detected. Saliva sample collection, lack of viral RNA extraction, and the capability for target multiplexing lends DISCoVER favorable properties for a point-of-care diagnostic. Further integration with a microfluidic platform and detection device will facilitate frequent testing for schools and workplaces as part of a robust infrastructure for pandemic surveillance.

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Author Contributions

As co-first authors, S.A., A.F. and S.S.C. designed and performed experiments, analyzed the data, and prepared the manuscript. P.D.H. conceived the project, designed experiments, co-supervised this work, and wrote the manuscript with input from all authors. D.F.S. and J.A.D. provided experimental input, edited the manuscript, and co-supervised this work. N.P. edited the manuscript and, with M.L. contributed to experimental design. D.C.S., T.Y.L., and G.J.K. purified proteins for assays and, with E.D. and S.K., provided biochemical expertise. A.M. developed computational methods for guide RNA selection with L.F.L., and S.B.B. and E.V.D. performed BSL-3 work with supervision from E.H. and S.S.

Competing Interests

The Regents of the University of California have filed patents related to this work. P.D.H. is a cofounder of Spotlight Therapeutics and serves on the board of directors and scientific advisory board, and is a scientific advisory board member to Vial Health and Serotiny. D.F.S. is a cofounder of Scribe Therapeutics and a scientific advisory board member of Scribe Therapeutics

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and Mammoth Biosciences. J.A.D. is a cofounder of Caribou Biosciences, Editas Medicine, Scribe Therapeutics, and Mammoth Biosciences. J.A.D. is a scientific advisory board member of Caribou Biosciences, Intellia Therapeutics, eFFECTOR Therapeutics, Scribe Therapeutics, Mammoth Biosciences, Synthego, Algen Biotechnologies, Felix Biosciences, and Inari. J.A.D. is a Director at Johnson & Johnson and has research projects sponsored by Biogen, Pfizer, AppleTree Partners, and Roche.

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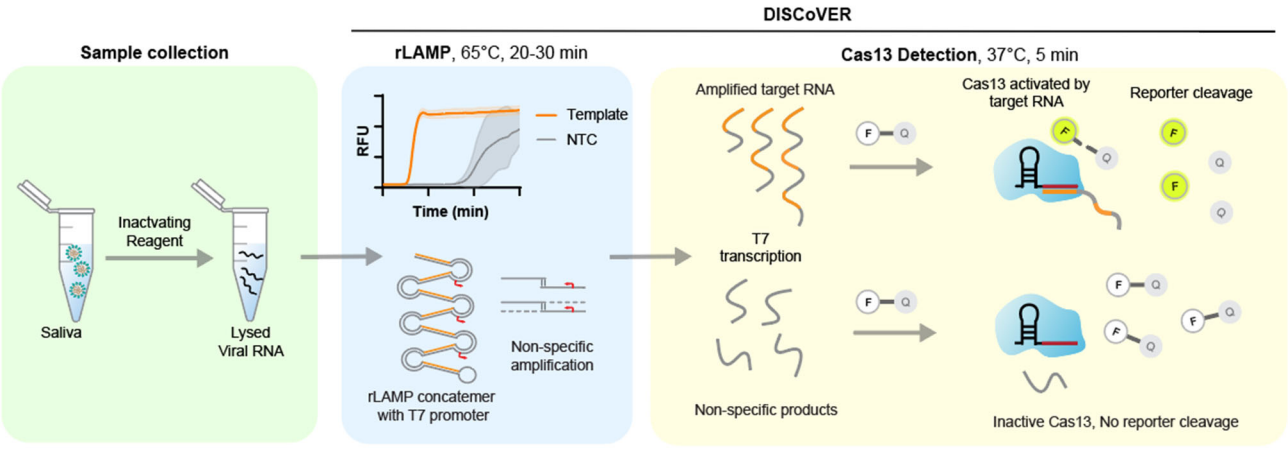


Figure 1. Schematic of DISCoVER sample to answer workflow. Saliva samples are collected in tubes containing buffers for direct RNase inactivation and viral lysis. The rLAMP (RNA transcription following LAMP) reaction employs two mechanisms for amplification of target SARS-CoV-2 RNA. Cas13 enzymes are programmed with a guide RNA to specifically recognize the desired RNA molecules over non-specifically amplified products. Subsequent activation of Cas13 ribonuclease activity results in cleavage of reporter molecules for saturated signal within 5 minutes of CRISPR detection, enabling rapid reporting of attomolar concentrations of SARS-CoV-2. By exploiting template switching and CRISPR programmability, the DISCoVER system can contribute to increased community surveillance of diverse pathogens.

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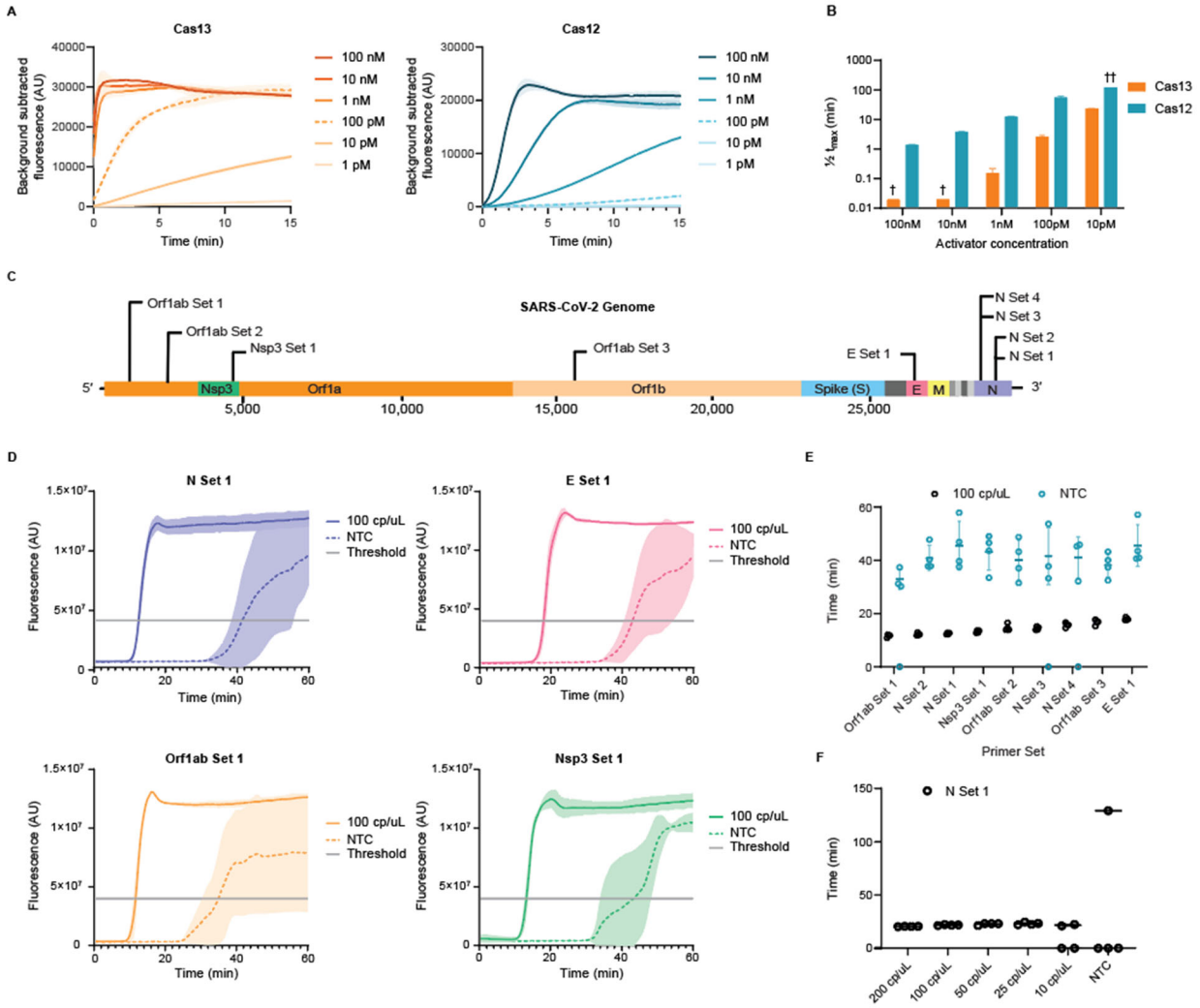


Figure 2. Direct nucleic acid detection with CRISPR-Cas enzymes and LAMP. A. Cas13 and Cas12 detection kinetics at varying activator concentrations. Values are mean ± SD with n = 3. **B.** Cas13 and Cas12 time to half-maximum fluorescence. †, Time to half maximum fluorescence was too rapid for reliable detection. ††, Time to half maximum fluorescence could not be determined within the 120 min assay runtime. Values are mean ± SD with n = 3. **C.** Schematic of SARS-CoV-2 genome sequence, with LAMP primer set locations indicated. **D.** Representative fluorescence plots of LAMP amplification of 100 copies/ µL of synthetic SARS-CoV-2 RNA or NTC. NTC, no-template control. Shaded regions denote mean ± SD with n = 3. **E.** Time-to-threshold of 9 screened LAMP primer sets, targeting synthetic SARS-CoV-2 RNA or NTC. Replicates that did not amplify are represented at 0 minutes. Error bars represent SD of amplified replicates. **F.** Limit of detection assay of LAMP using N Set 1 primer set. Replicates that did not amplify are represented at 0 minutes. Error bars represent SD of amplified technical replicates.

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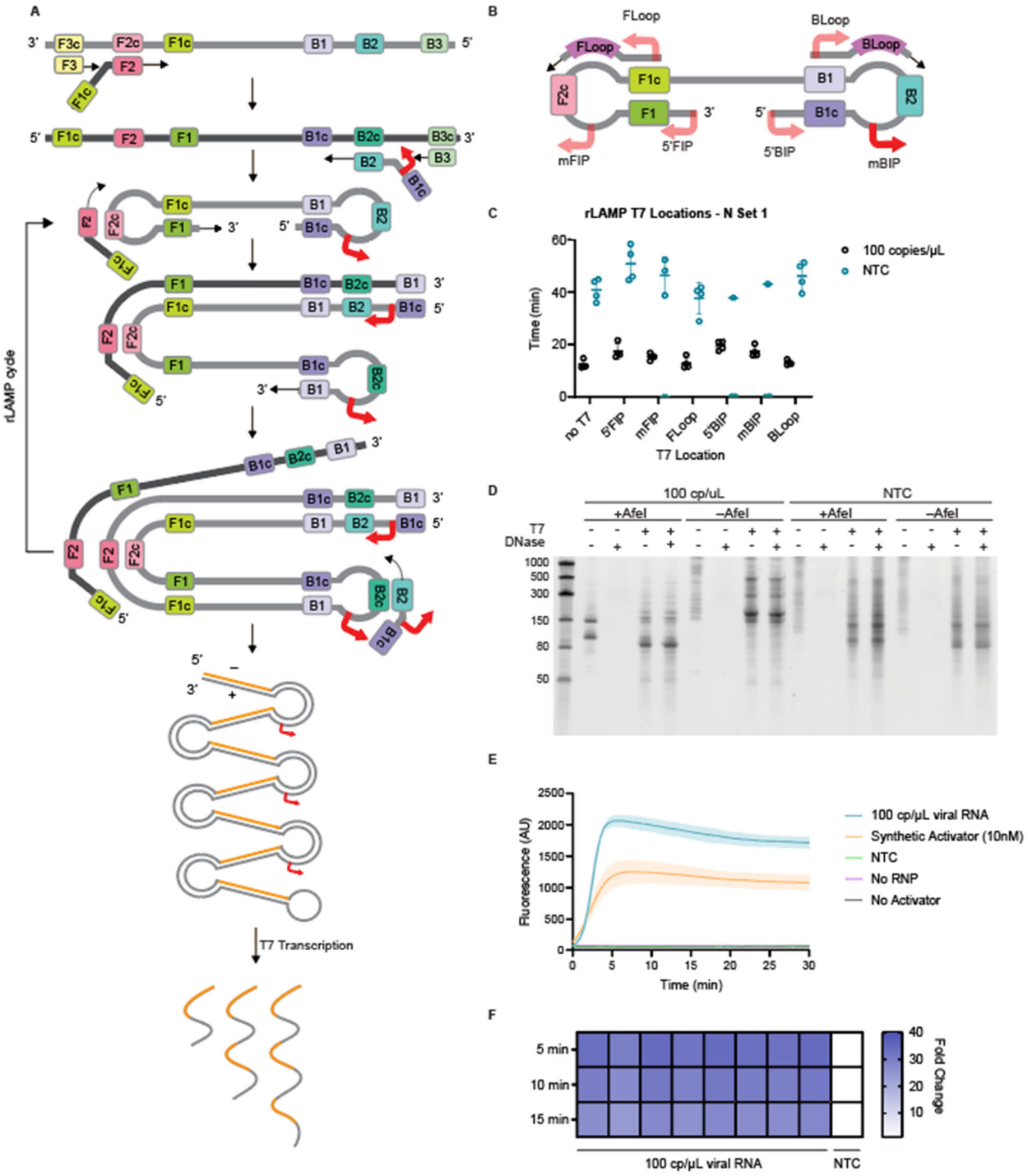


Figure 3. Development of RNA transcription following LAMP (rLAMP) for two layers of nucleic acid amplification. A. Schematic of rLAMP mechanism for exponential DNA amplification using F3/B3 and FIP/BIP primers, resulting in higher-order inverted repeat structures. Red arrows indicate location of T7 promoter sequence, inserted in the mBIP primer. Upon T7 transcription, the resulting RNA contains one or more copies of the Cas13 crRNA

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target sequence (orange). **B**. Schematic of the location of different T7 promoter locations on the rLAMP dumbbell structure and loop primers. **C**. rLAMP time to threshold of 6 distinct T7 promoter insertions. Replicates that did not amplify are represented at 0 minutes. Error bars represent SD of amplified technical replicates. **D**. Denaturing PAGE gels of mBIP rLAMP products to verify successful T7-mediated transcription. AfeI cleaves in the crRNA target region of templated products, which is expected to result in a single major transcribed species. **E**. Kinetics of T7 transcription and Cas13 detection on mBIP rLAMP products. RNP, ribonucleoprotein. Values are mean ± SD with n = 3. **F**. Cas13 detection of 8 technical replicates of mBIP rLAMP amplification on genomic RNA, depicted as fold-change over NTC at different reaction end-points.

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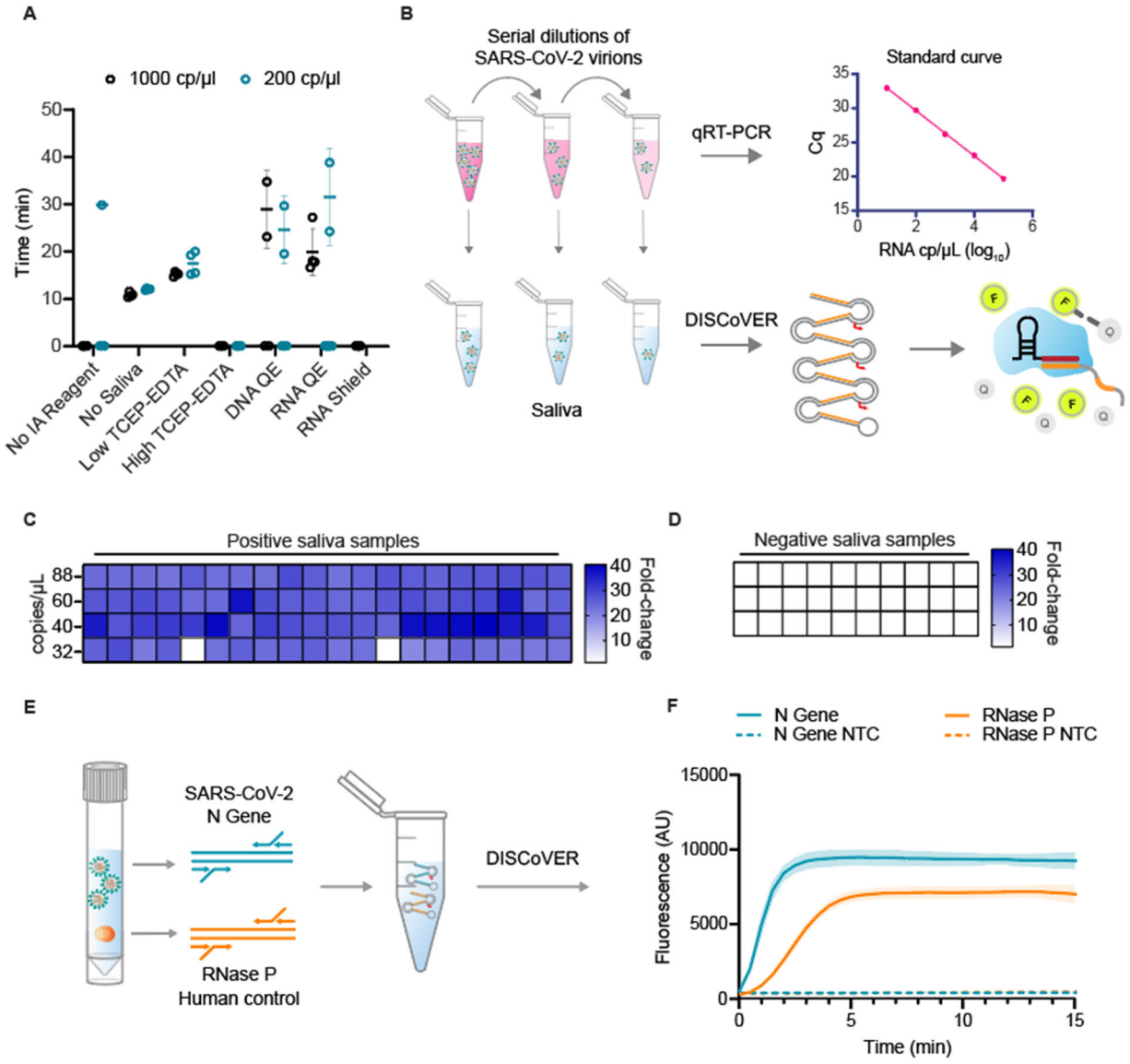


Figure 4. Development of DISCoVER for extraction-free saliva detection. **A**. Direct saliva lysis conditions were tested for compatibility with the DISCoVER workflow. Replicates that did not amplify are represented at 0 minutes. Error bars represent SD of amplified technical replicates. IA: inactivation reagent; QE: QuickExtract. **B**. Schematic of contrived saliva sample generation, quantification via qRT-PCR, and detection via DISCoVER to determine analytical sensitivity. **C.** Fold-change in DISCoVER signal relative to NTC on SARS-CoV-2 positive saliva samples at 5 minutes of Cas13 detection. **D**. Fold-change in DISCoVER signal relative to NTC on 30 negative saliva samples collected before November 2019, at 5 minutes of Cas13 detection. **E.** Schematic of rLAMP multiplexing with SARS-CoV-2 (N Gene) and human internal control (RNase P) primer sets. **F**. DISCoVER signal of SARS-CoV-2 positive saliva samples after multiplexed rLAMP. Values are mean ± SD with n = 3.

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